Casein Components Soluble in Chloroform-Methanol (2:1) and in Fifty Per Cent Aqueous Ethanol

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Abstract

Chloroform-methanol (2:1) extracted approximately 2% of protein from acid precipitated casein. This soluble fraction was identical qualitatively and quantitatively with the soluble casein fraction obtained by 50% aqueous ethanol extraction. γ-Casein and temperature-sensitive casein are the major components in the soluble casein fraction. Six or more minor components, including three in the λ-casein group, also are present.

Casein for laboratory use is usually prepared from skimmed cow's milk by acid precipitation, at pH 4.7, then washed with water and fat solvents (alcohol, acetone, and ether) (11). Practically all research, up to this time, has been directed at the insoluble product.

Osborne and Wakeman (9) reported that 50 to 70% ethanol solubilized a small proportion (approximately 0.8%) of casein. El-Negoumy (4) reported that part of the β- and γ-caseins was removed by washing, when the casein had been treated with NaOH, even at pH 7.0. Using the method of Folch et al. (5), for the isolation and purification of total lipids from various milk fractions, Cerbulis observed that chloroform-methanol (2:1, v/v) extracted an appreciable amount of nondialyzable proteins from casein (3).

In our studies the amount and composition of nondialyzable material obtained by the method of Folch et al. (5) from the casein fraction of bovine milk have been determined. For convenience, this material will be designated as soluble casein. This soluble casein fraction was compared electrophoretically with the soluble casein fraction obtained by the extraction with 50% aqueous ethanol (9).

Experimental Procedure

Preparation of casein fractions. Casein from milk of Holstein cows was prepared as described previously (3). Casein was washed repeatedly with distilled water, adjusting the suspension to pH 4.7; then the casein was treated as follows:

A. *Extraction with chloroform-methanol.* The casein was freeze-dried. The casein was extracted with chloroform-methanol (2:1, v/v) as described previously (3), using 20 ml of solvent per gram of freeze-dried casein. The samples were extracted three times by continuous stirring with the solvent for 3-4 hr. The extracts were evaporated in vacuo to dryness, the residue taken up with a small volume of chloroform-methanol (2:1), and washed with 0.2 vol of water, as described by Folch et al. (5). The chloroform layer contained lipids (neutral lipids and phospholipids). The wash-water with interphase solids was dialyzed against distilled water for four days, changing water three or four times daily.

B. *Extraction with ethanol.* The wet casein (100 g, 80% moisture content) was mixed with 500 ml 60% aqueous ethanol and stirred for 2 hr, then filtered. The second extraction was performed with 50% aqueous ethanol. The extracts were combined, evaporated in vacuo to 100 ml, then dialyzed against distilled water as described above, and the nondialyzable fraction was freeze-dried and used for further studies.

Thin-layer chromatography (TLC). The technique was described previously (3). Silica gel G was used throughout the experiments. The developing solvents were petroleum ether–diethyl ether–acetic acid, 90:10:1 (v/v) for neutral lipids, chloroform-methanol-water–acetic acid, 65:25:4:2 (v/v) for phospholipids, and n-butanol-pyridine–acetic acid–water, 30:20:6:24 (v/v) for nondialyzable proteins. Iodine vapor was used for general staining, ninhydrin (8) for proteins, and p-anisidine (2) for carbohydrates.

Polyacrylamide gel electrophoresis. Electrophoresis was performed in a vertical cell, using Tris-EDTA-borate buffer at pH 8.6. The gel was prepared with an acrylamide concentration of 7% and a urea molarity of 4.5 (10). Mercaptoethanol was used (13) in some experiments, but it has no influence on the number of the bands obtained. The gel was dyed with Amido Black staining solution (10).

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Results

Content of soluble caseins. Extraction by chloroform-methanol was practically completed in two extractions: the first extraction solubilized 85%; the second extract, 10% of the soluble caseins, and the third extract, 5%.

The same yield was obtained, using either chloroform-methanol or 50% aqueous alcohol as extractant, i.e., approximately 2% by weight of pooled milk casein (on dry weight basis). This is equivalent to 0.6 g of soluble casein per liter of pooled whole milk. Chloroform-methanol extracts were also prepared from the caseins from ten cows of several breeds. The average content of soluble caseins was approximately 1.9% (1.7-2.3% for eight of the samples); one sample contained 5% and another sample contained 0.02% only.

Casein of one sample of pooled goat milk contained only 0.35% of soluble casein.

Lactose in soluble casein. TLC and paper chromatography showed that the soluble casein fraction contained a small amount of lactose.

Hydrolysis of the soluble casein fraction (after two days of dialysis) with 1 M H_2SO_4 at 100 C for 2 hr showed the presence of glucose and galactose in 1:1 ratio. No other sugars were observed. The lactose was strongly associated with the caseins, although it was not covalently bound. Dialysis, changing water 3-4 times daily, eliminated the lactose slowly; usually, dialysis for 4-5 days was necessary to remove all lactose.

Phenol treatment (12) of soluble casein revealed lactose as the only associated sugar. The 3.6 g of soluble casein was mixed with 100 ml phenol and 100 ml H_2O, stirred for 30 min at 65-70 C, then cooled and centrifuged. The phenol layer contained the peptide moiety and the water layer contained sugars. The water layer was evaporated to dryness and studied for sugars by paper chromatography.

Lipid content of soluble casein. The soluble casein fraction, after dialysis and freeze-drying, was not completely soluble in the same chloroform-methanol (2:1) solvent, due to physical changes in the dry residue. The freeze-dried soluble casein gave a positive reaction for lipids with Rhodamine 6G. Extraction of the soluble casein fraction with chloroform (four times, 24 hr) yielded up to 0.5% lipids. TLC showed that this lipid fraction contained neutral fat (triglycerides, diglycerides, monoglycerides, cholesterol, free fatty acids, and unidentified components) and phospholipids (cephalin, lecithin, sphingomyelin, and traces of two other lipids).

Chemical analysis. Freeze-dried soluble casein fraction contained 12.49% nitrogen and 0.29% phosphorus.

Electrophoresis of soluble caseins. According to criteria previously stated (6, 7), the major components obtained by chloroform-methanol extraction of whole casein were γ-casein and temperature-sensitive casein. When subjected to gel electrophoresis, the soluble casein fraction was resolved into two major bands, together with six or more weaker bands (Fig. 1, 3). Verification of the identity of the two major bands as γ- and temperature-sensitive caseins was obtained by gel electrophoresis of the soluble casein, placed side by side with known samples of γ- and temperature-sensitive casein (Fig 1, 2 and 3). The minor bands did not coincide with any known milk proteins like α-casein, β-casein, κ-casein β-lactoglobulin, or α-lactalbumin. α-Casein and β-casein were not soluble in chloroform-methanol (2:1), but whey proteins were somewhat soluble. Bands of the soluble whey proteins did not coincide with the bands of the soluble caseins.

Almost identical electrophoresis patterns were obtained for all preparations. Variations were observed only in the intensity of minor components.

Column chromatography. The soluble casein fraction (0.45 g) was fractionated on a DEAE-cellulose column, as described by Groves et al. (6). Protein recovery was 90%, on a dry weight basis. Electrophoretic patterns of the fractions obtained through column chromatography are shown in Fig. 1, 4-8. The yields were: 4) 0.03, 5) 0.11, 6) 0.25, 7) 0.02 g, respectively.

Discussion

Extraction of casein with chloroform-methanol (2:1), a common lipid solvent, and with 50% aqueous ethanol was found to extract approximately 2% protein from acid-precipitated casein. The extracted protein was found to be γ-casein, temperature-sensitive casein, and at least six minor casein components [including three in the region of the λ-casein group (1)].

Two to three extractions by chloroform-methanol (2:1), or by 50% aqueous ethanol, completely extracted the soluble casein fraction. The extraction by ethanol was more convenient, because the acid-precipitated casein did not need to be freeze-dried before extraction.

Results described in this paper suggest that the extraction of lipids of casein with chloroform-methanol (2:1) and the extraction of casein with 50% aqueous ethanol are simple methods for separating the above-mentioned minor components from casein.

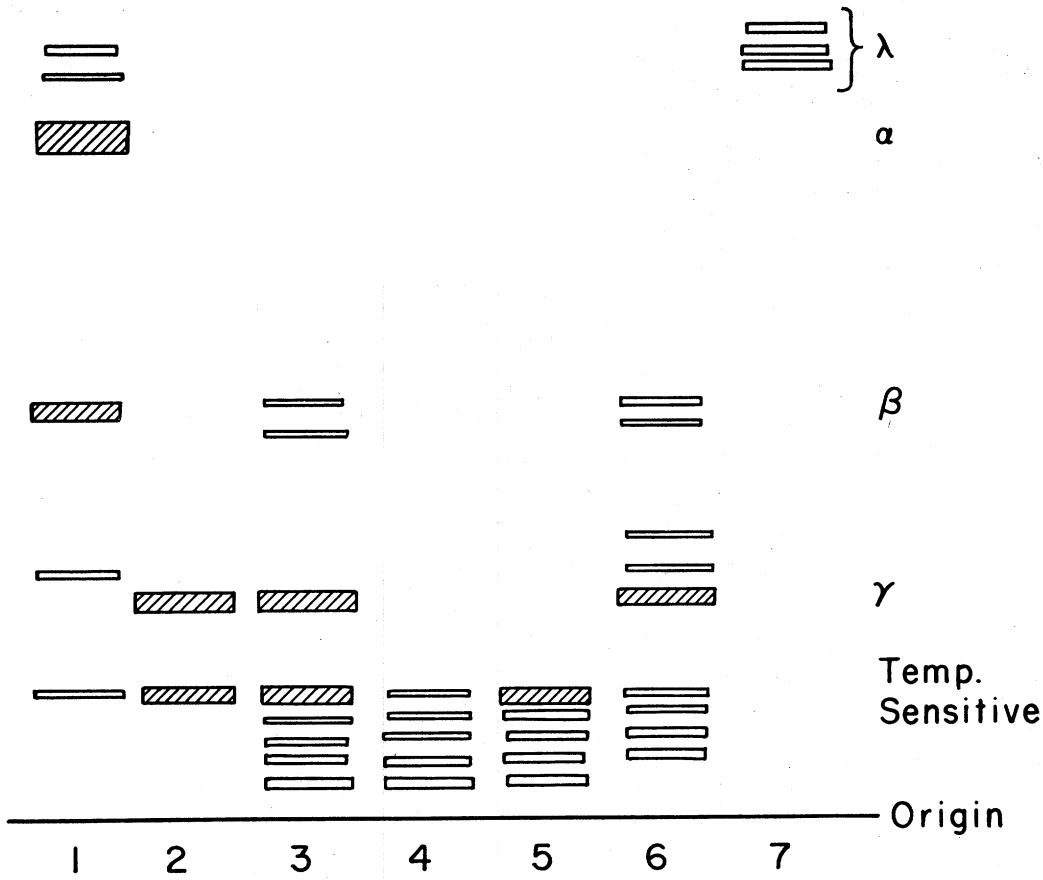

FIG. 1. Polyacrylamide-gel electrophoresis patterns of caseins: 1) Acid-precipitated whole casein; 2) γ- and temperature-sensitive caseins; 3) soluble casein fraction; 4-7) soluble casein fraction partially separated on DEAE column (6).

The casein minor components also contained phosphorus. The lipid content of 0.5% could not contribute significantly to the phosphorus content of the soluble casein fraction. The fact that γ- and temperature-sensitive caseins are soluble in a lipid extractant, may point to properties that should be considered in any future investigation regarding the nature of these proteins.

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References

1) Brunner, J. R. 1966. Personal communication.

2) Cerbulis, J. 1955. Paper Chromatography of Sugar Alcohols and Their Glycosides. Anal. Chem., 27: 1400.

(3) Cerbulis, J. 1967. Distribution of Lipids in Various Fractions of Cow's Milk. J. Agr. Food Chem. (In press.)

(4) El-Negoumy, A. M. 1963. Effect of Method of Preparing Isoelectric Casein on Its Electrophoretic Properties. J. Dairy Sci., 46: 768.

(5) Folch, J., Lees, M., and Sloane Stanley, G. H. 1957. A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. J. Biol. Chem., 226: 497.

(6) Groves. M. L., McMeekin, T. L., Hipp, N. J., and Gordon, W. G. 1962. Preparation of β- and γ-Caseins by Column Chromatography. Biochim. Biophys. Acta, 57: 197.

(7) Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L. 1950. Separation of γ-Casein. J. Am. Chem. Soc., 72: 4928.

(8) Levy, A. L., and Chung, D. 1953. Two-Dimensional Chromatography of Amino

Acids on Buffered Papers. Anal. Chem., 25: 396.

(9) Osborne, T. B., and Wakeman, A. J. 1918. Some New Constituents of Milk. III. A New Protein, Soluble in Alcohol. J. Biol. Chem., 33: 243.

(10) Thompson, M. P., Kiddy, C. A., Johnston, J. O., and Weinberg, R. M. 1964. Genetic Polymorphism in Casein of Cows' Milk. II. Confirmation of the Genetic Control of β-Casein Variation. J. Dairy Sci., 47: 378.

(11) Warner, R. C. 1944. Separation of α- and β-Casein. J. Am. Chem. Soc., 66: 1725.

(12) Westphal, O., Lüderitz, O., and Bister, F. 1952. Extraction of Bacteria with Phenol/Water. Z. Naturforsch., 7b: 148.

(13) Woychik, J. H. 1965. Preparation and Properties of Reduced κ-Casein. Arch. Biochem. Biophys., 109: 542.